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Design of Small-Molecule Peptidic and Nonpeptidic Smac Mimetics

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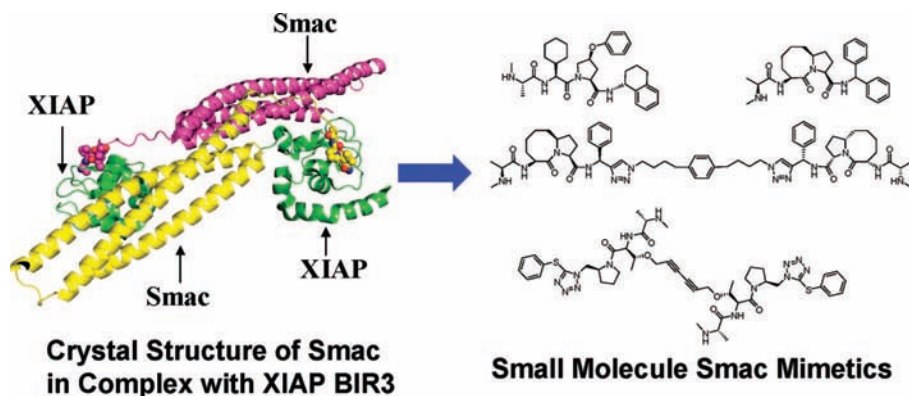
Crystal Structure of Smac in Complex with XIAP BIR3

Small Molecule Smac Mimetics

Smac/DIABLO is a protein released from mitochondria into the cytosol in response to apoptotic stimuli. Smac promotes apoptosis at least in part through antagonizing inhibitor of apoptosis proteins (IAPs), including XIAP, cIAP-1, and cIAP-2. Smac interacts with these IAPs via its N-terminal AVPI binding motif. There has been an enormous interest in academic laboratories and pharmaceutical companies in the design of small-molecule Smac mimetics as potential anticancer agents. This task is particularly challenging because it involves targeting protein–protein interactions. Nevertheless, intense research has now generated potent, specific, cell-permeable small-molecule peptidomimetics and nonpeptidic mimetics.

To date, two types of Smac mimetics have been reported, namely, monovalent and bivalent Smac mimetics. The monovalent compounds are designed to mimic the binding of a single AVPI binding motif to IAP proteins, whereas the bivalent compounds contain two AVPI binding motif mimetics tethered together through a linker. Studies from several groups have clearly demonstrated that both monovalent and bivalent Smac mimetics not only enhance the antitumor activity of other anticancer agents but also can induce apoptosis as single agents in a subset of human cancer cell lines *in vitro* and are capable of achieving tumor regression in animal models of human cancer. In general, bivalent Smac mimetics are 100–1000 times more potent than their corresponding monovalent Smac mimetics in induction of apoptosis in tumor cells. However, properly designed monovalent Smac mimetics can achieve oral bioavailability and may have major advantages over bivalent Smac mimetics as potential drug candidates.

In-depth insights on the molecular mechanism of action of Smac mimetics have been provided by several independent studies. It was shown that Smac mimetics induce apoptosis in tumor cells by targeting cIAP-1/-2 for the rapid degradation of these proteins, which leads to activation of nuclear factor κB (NF-κB) and production and secretion of tumor necrosis factor α (TNFα). TNFα promotes formation of a receptor-interacting serine–threonine kinase 1 (RIPK1)-dependent caspase-8-activating complex, leading to activation of caspase-8 and -3/-7 and ultimately to apoptosis. For the most efficient apoptosis induction, Smac mimetics also need to remove the inhibition of XIAP to caspase-3/-7. Hence, Smac mimetics induce apoptosis in tumor cells by targeting not only cIAP-1/-2 but also XIAP. The employment of potent, cell-permeable, small-molecule Smac mimetics has yielded important insights into the regulation of apoptosis by IAP proteins. To date, at least one Smac mimetic has been advanced into clinical development. Several other Smac mimetics are in an advanced preclinical development stage and are expected to enter human clinical testing for the treatment of cancer in the near future.

Introduction

Apoptosis, or programmed cell death, is a critical cellular process in normal development and homeostasis of multicellular organisms. Inappropriate regulation of apoptosis is associated with many human diseases, including cancer,[1−23] and it is now recognized that one hallmark of cancer cells is their compromised ability to undergo apoptosis.[2] Targeting critical apoptosis regulators is an attractive strategy for the development of new classes of therapies for the treatment of cancer and other human diseases.[1−23]

The inhibitor of apoptosis proteins (IAPs) are a class of central apoptosis regulators, although their role is not limited to apoptosis (Figure 1).[4,5] The X-linked inhibitor of apoptosis protein (XIAP) is perhaps the best characterized member of all the IAPs.[5] It effectively suppresses apoptosis at least in part by binding to and inhibition of three members of the caspase family of enzymes, the two effectors, caspase-3 and -7, and an initiator, caspase-9.[6] XIAP contains three baculoviral IAP repeat (BIR) domains. The third BIR domain (BIR3) of XIAP selectively targets caspase-9,[7,8] whereas the BIR2 domain, together with the linker preceding BIR2, inhibits both caspase-3 and caspase-7.[9−11] Consistent with its potent apoptosis inhibitory function, XIAP is found to be highly expressed in many human tumor cell lines and tumor samples from patients[12] and plays an important role in the resistance of cancer cells to a variety of anticancer drugs.[13]

Smac/DIABLO is a protein released from mitochondria into the cytosol in response to apoptotic stimuli.[14,15] Smac functions as an endogenous inhibitor of XIAP and other IAP proteins (Figure 1).[6] As a dimer,[16] Smac targets both the BIR2 and BIR3 domains in XIAP concurrently and prevents the inhibition of XIAP not only to caspase-9 but also to caspase-3/-7.[6] It blocks the inhibition of XIAP to caspase-9 by binding to the BIR3 domain in XIAP through its AVPI tetra-peptide binding motif and competing directly with a similar ATPF tetrapeptide in caspase-9.[6,17,18] The mechanism by which Smac removes the inhibition of XIAP to caspase-3/-7 is not completely clear, but it has been proposed that the interaction of Smac protein through its AVPI motif with the BIR2 domain of XIAP also prevents the binding and inhibition of XIAP to caspase-3/-7.[19,21]

Because XIAP blocks apoptosis at the downstream effector phase, a point where multiple signaling pathways converge, it represents a particularly attractive molecular target for the design of new classes of anticancer drugs aimed at overcoming the apoptosis resistance of cancer cells.[5,13] Several strategies have been employed for the design of small-molecular inhibitors of XIAP. One approach has been to block the interaction between XIAP BIR2 and caspase-3/-7. Employing high-throughput screening and parallel solid-phase synthesis, Wu and colleagues identified a series of small-molecule inhibitors of the caspase-3/XIAP interaction.[22] Schimmer and colleagues discovered a class of polyphenylureas by chemical library screening and showed that by antagonizing XIAP and with a mechanism of action different from that of Smac protein, these compounds can restore the activities of caspase-3 and -7, but not that of caspase-9.[23] Another strategy is to design small molecules to mimic the Smac AVPI binding motif; this has attracted a great deal of attention in recent years and is the focus of this Account.

Structural Basis for the Design of Smac Mimetics

The crystal structure of Smac/DIABLO protein in a complex with the XIAP BIR3 domain has been determined by Shi's group,[17] and the solution structure of Smac peptide complexed with the BIR3 domain was established by Fesik and his colleagues at Abbott Laboratories.[18]

The crystal structure showed that Smac protein forms an elongated homodimer (Figure 2A).[17] Both crystal and NMR solution structures[17,18] clearly revealed that the N-terminal four residues (Ala1-Val2-Pro3-Ile4) in Smac recognize and bind to a surface groove on XIAP BIR3 (Figure 2B). The methyl group of the Ala1 residue inserts into a small hydrophobic pocket; the free amino group forms strong hydrogen bonds to the Glu314 and Gln319 residues on BIR3, and the backbone carbonyl group forms a suboptimal hydrogen bond to the indole NH group in Trp323. The amino and carbonyl groups of Val2 form optimal hydrogen bonds with the carbonyl and amino groups of Thr308, respectively, while the Val2 side chain, with no interactions with protein residues, is exposed to solvent. The five-membered ring of Pro3 has van der Waals contacts with the side chains of Trp323 and Tyr324, and
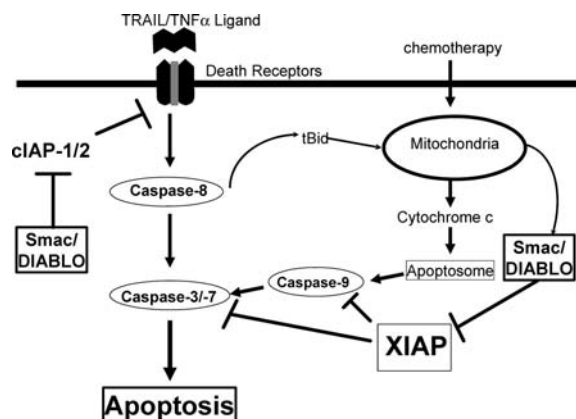

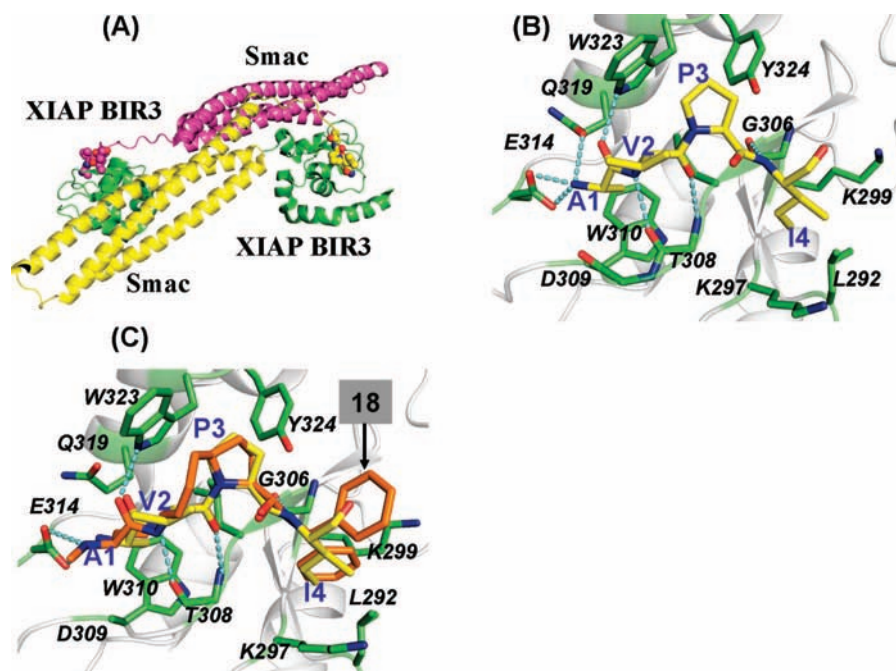**FIGURE 1.** A simplified apoptotic pathway.

FIGURE 2. (A) Crystal structure of Smac in complex with XIAP BIR3 protein, (B) detailed interactions between the AVPI binding motif and XIAP BIR3 residues, and (C) crystal structure of compound **18** in complex with XIAP BIR3 (Protein Data Bank access code 2JK7), in superposition to that of Smac AVPI peptide in complex with XIAP BIR3.
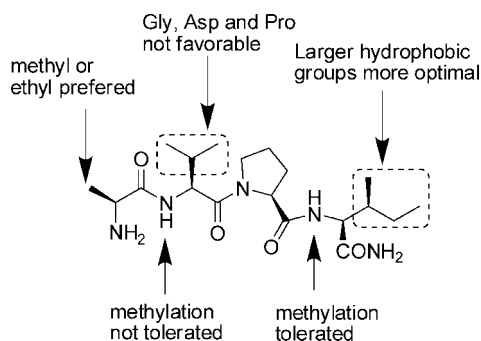

FIGURE 3. Summary of structure−activity relationship of Smac-based peptides to XIAP BIR3.

finally, the amino group of the Ile4 residue forms a hydrogen bond with the carbonyl group of Gly306, and its hydrophobic side chain inserts into a hydrophobic pocket formed by the side chains of Leu292 and Val298 and the hydrophobic portion of the side chains in Lys297 and Lys299. Such structural information at the atomic level has been the basis for the design of both peptidic and nonpeptidic Smac mimetics.

Design of Smac-Based Peptides

McLendon's group has carried out extensive modifications to the AVPI tetrapeptide and obtained a comprehensive structure−activity relationship (SAR) of Smac-based peptides binding to the XIAP BIR3 domain (Figure 3).[24]

The AVPI peptide binds to XIAP BIR3 with a K_d value of 480 nM, but replacement of the Ala1 residue by a glycine or serine residue results in a more than 20-fold loss in binding affinity, while a slight improvement in binding affinity is achieved with the unnatural amino acid 2-aminobutyric acid. Replacement of the valine in AVPI shows that this position can tolerate many other amino acid residues without significant reduction in binding affinity. This is consistent with the experimental structural information, which shows that the side chain of valine has no close contacts with protein residues and is exposed to solvent. Replacement of the valine residue by aspartate, glycine, or proline, however, results in significant loss in binding affinity. Modifications of the fourth residue, isoleucine, indicate that a hydrophobic residue such as valine, phenylalanine, tryptophan, or leucine is highly preferred with phenylalanine being the most preferred residue. A charged or polar residue such as lysine, arginine, glutamate, aspartate, histidine, glutamine, and asparagine at this position is detrimental to binding. Modification at the peptide bond between residues 1 and 2 by methylation disrupts a structurally important hydrogen bond and has a large negative effect on binding, but N-methylation of residue 4 has a much smaller effect. The structure−activity relationship obtained from this study has provided a very useful guide to the design of peptidic- and nonpeptidic mimetics of Smac.

Smac-based peptides have potent binding affinities to XIAP, but they are not cell-permeable. To address this limitation, a number of early studies employed a strategy to tether a car-
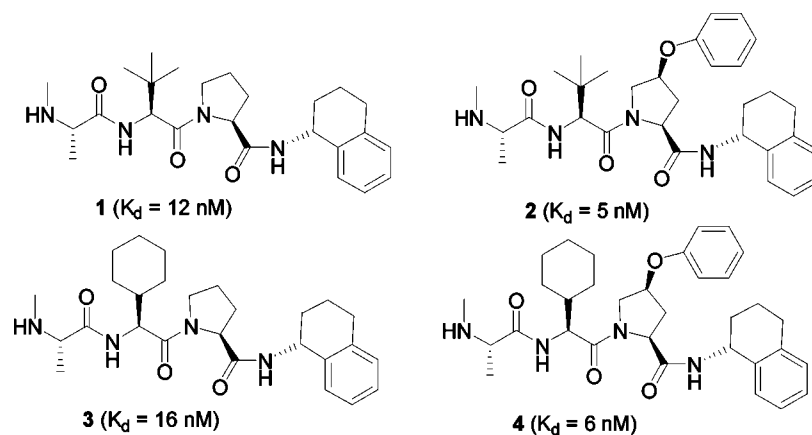
FIGURE 4. Representative potent Smac peptidomimetics.

rier peptide to a Smac-based peptide so facilitating intracellular delivery.[25–27] It was shown that these relatively cell-permeable Smac-based peptides can sensitize various tumor cells *in vitro* to the antitumor activity of Apo-2L/TNF-related apoptosis-inducing ligand (TRAIL), as well as chemotherapeutic agents such as paclitaxel, etoposide, SN-38, doxorubicin, and cisplatin.[26] Furthermore, one cell-permeable Smac-based peptide was shown to strongly enhance the antitumor activity of Apo-2L/TRAIL in an intracranial malignant glioma xenograft model *in vivo*, achieving complete eradication of established tumors.[25] Similarly, another cell-permeable Smac-based peptide in combination with cisplatin was shown to regress tumor growth *in vivo* in H460 non-small-cell lung cancer xenografts with little toxicity to the mice.[27] Of note, these cell-permeable peptides were injected directly into the tumors in both *in vivo* studies. Nevertheless, these early studies provide the important proof-of-the-concept that cell-permeable Smac peptidomimetics and nonpeptidic mimetics may be useful as new cancer therapies, especially when used in combination with other anticancer drugs.

Design of Smac Peptidomimetics

Oost and colleagues at Abbott Laboratories have carried out extensive chemical modifications of the AVPI peptide in an effort to derive potent Smac peptidomimetics.[28] Chemical modifications of the Ala1 residue using different amino acids showed that the natural methyl or an ethyl side chain are most preferred for binding, consistent with the data obtained by McLendon's group.[24] Substitution of the free terminal amino group by one methyl group is well tolerated, but dimethylation decreases the binding to XIAP BIR3 by a factor of >100. Consistent with the previous report,[24] modifications of the Val2 showed that this residue can be replaced by many other residues without a significant loss of the binding affinity, although replacement by glycine leads to a >30-fold loss

of binding affinity. In addition, it was shown that an L-configuration of this residue is essential for binding. Replacement of the five-membered ring in the Pro3 residue with four- or six-membered rings results in a 5–7-fold loss in binding affinity and a greater loss with other residues. Introduction of a hydrophobic group to the five-membered ring in Pro3 can slightly improve the binding affinity. Modifications to the Ile4 residue showed that a variety of hydrophobic entities are tolerated, phenylanaline and phenylglycine being the most preferred. Among all the compounds designed and evaluated, compounds **1**, **2**, **3**, and **4** have the highest binding affinities to XIAP BIR3 with K_d values of 12, 5, 16, and 6 nM, respectively (Figure 4).

These potent Smac peptidomimetics have been shown to be effective in rescuing XIAP BIR3-mediated inhibition of caspase activity in a fully reconstituted functional assay containing Apaf-1, procaspase-9 and procaspase-3, dATP, and cytochrome *c*. Compounds **1**, **2**, and **3** achieve EC_{50} values of 0.29, 0.24, and 0.31 μM, respectively, in recovering the caspase activity. Such functional data provide direct evidence for their functional antagonism against XIAP BIR3.

These potent Smac peptidomimetics are also effective in induction of caspase-3 activation in the MDA-MB-231 human breast cancer cell line. Compounds **1** and **3** potently inhibit cell growth with IC_{50} values of 68 and 13 nM, respectively, in the MDA-MB-231 cell line and effectively induce cell death. Compound **3** also demonstrates modest activity in inhibition of tumor growth in the MDA-MB-231 xenografts in mice.

These *in vitro* and *in vivo* data obtained using potent and cell-permeable Smac peptidomimetics provide important evidence that Smac mimetics may have a therapeutic potential as single agents for the treatment of human cancer in a subset of human cancers.
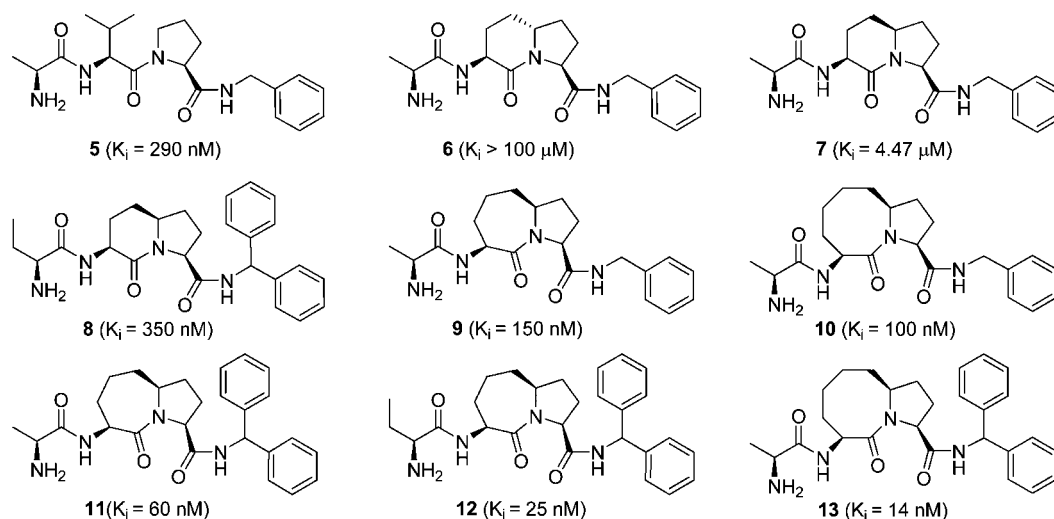
FIGURE 5. Conformationally constrained nonpeptidic Smac mimetics.

Design of Conformationally Constrained Smac Mimetics

The Wang laboratory from the University of Michigan was the first to report the design of conformationally constrained, bicyclic Smac mimetics using a structure-based strategy (Figure 5).[29–32]

The experimental structures of Smac protein/peptide in complex with XIAP BIR3 show that the isopropyl group of Val2 is exposed to the solvent and has no specific interaction with the XIAP BIR3 protein, while the five-membered ring of Pro3 has hydrophobic contacts with Trp323 in XIAP BIR3. Based upon modeling predictions, it was proposed that these two residues can be fused together to form a bicyclic lactam structure without seriously altering the conformation of the AVPI peptide bound to XIAP BIR3. Cyclization of the side chains of Val2 and Pro3 produces a new chiral center and in order to determine which configuration of the chiral center is desirable for binding to XIAP BIR3, two stereoisomers, **6** and **7**, each containing the [6,5] bicyclic ring structure were designed and synthesized.[29,30] While **7** has a K_i value of 4.47 μM to XIAP BIR3 protein, **6** shows a K_i value >100 μM, indicating that the *R* configuration for the chiral center is much better suited to binding to XIAP BIR3. With SAR data obtained from peptidic Smac mimetics, compound **8** was designed and shown to have a K_i of 350 nM, as potent as the natural Smac AVPI peptide.

Compound **7** containing the [6,5] bicyclic lactam structure is 8 times less potent than the AVPI peptide and 15 times less than its corresponding peptidic mimetic **5**. Modeling revealed that although compound **7** can largely mimic both **5** and the Smac AVPI peptide for binding to XIAP, the binding is less than optimal. Expansion of the [6,5] bicyclic ring system in com-

TABLE 1. Binding Affinities of Bicyclic Smac Mimetics to XIAP BIR3 Protein and Cell Growth Inhibition in the MDA-MB-231 Cancer Cell Line

$X = (CH_2)_{1-2}$

	X	R_1	R_2	binding affinity to XIAP BIR3 (K_i, μM)	cell growth inhibition (IC_{50}, μM)
11 (SM-104)	CH_2	NH_2	Me	0.060	>10
12 (SM-102)	CH_2	NH_2	Et	0.025	>10
13 (SM-128)	$(CH_2)_2$	NH_2	Me	0.014	>10
14 (SM-131)	CH_2	CH_3NH	Et	0.061	0.1
15 (SM-160)	CH_2	$N(CH_3)_2$	Et	14.4	3.0
16 (SM-161)	CH_2	OH	Et	29.0	70.0
17 (SM-122)	$(CH_2)_2$	CH_3NH	Me	0.026	0.26
18 (SM-130)	$(CH_2)_2$	CH_3NH	Et	0.067	0.41

pound **7** to either a [7,5] or an [8,5] ring system results in compounds that mimic the bound conformation of the AVPI peptide to XIAP more closely and show improved binding affinities.[30–32] This leads to the design of compounds **9** and **10**, which bind to XIAP BIR3 with K_i values of 150 and 100 nM, respectively, 30 or 45 times more potent than compound **7**. With SAR data obtained for [6,5] ring containing Smac mimetics, compounds **11**, **12**, and **13** were designed and shown to have K_i values of 60, 25, and 14 nM, respectively.

Although compounds **11**, **12**, and **13** achieve high binding affinities to XIAP BIR3, they were found to have very weak activities in cell-based assays in both cell growth inhibition and apoptosis induction in sensitive cancer cell lines, such as the MDA-MB-231 breast cancer cell line (Table 1). Their weak cellular activity suggests that they are not very cell-permeable, and it was hypothesized that the primary amine group in

these compounds may have a detrimental effect on their cell permeability or metabolic stability. To test this idea, compounds **14**, **15**, and **16** (Table 1) were designed by replacing the primary amino group in compound **12** with a secondary or tertiary amine or a hydroxyl group.[31,32] Evaluation of their binding affinities to XIAP BIR3 indicated that replacement of the primary amine group with an *N*-methyl amine decreases the binding affinity by a factor of 2 (**14** vs **12**), but replacement of the primary amino group with a dimethylamine (**15**) or a hydroxyl (**16**) weakens the binding affinity by 600 or 1000 times, respectively. Hence, the binding data showed that the primary and secondary amino groups are highly preferred at this site for Smac mimetics to achieve high binding affinities to XIAP BIR3 protein.

These compounds were tested for their activity in inhibiting cell growth in the MDA-MB-231 human breast cancer cell line. Although **12** and **14** have comparable binding affinities to XIAP BIR3, they display drastically different cellular activities. Compound **12** has very weak activity in inhibition of cell growth with an IC$_{50}$ of 50 μM (Table 1), but **14** achieves an IC$_{50}$ value of 0.1 μM and is thus 500 times more potent than **12**. Interestingly, although **15** has a much weaker binding affinity than **12** to XIAP, it is more potent than **12** in the cell growth inhibition assay (IC$_{50}$ = 3.0 μM). Compound **16** with a hydroxyl group has an IC$_{50}$ value of 70 μM, consistent with its very weak binding affinity to XIAP BIR3. Furthermore, replacement of the primary amino group with an *N*-methyl-amino group in compound **13** containing the [8,5] ring (compound **17**, Table 1) also dramatically improves its cellular activity.

Additional modifications of compound **17** yielded **19** and **20** (Figure 6).[32] Compounds **19** and **20** have a K_i value of 14 and 15 nM to XIAP BIR3 protein, respectively. Both compounds potently inhibit cancer cell growth in the MDA-MB-231 and other cancer cell lines.[32]

To obtain a solid structural basis for the interaction of these designed bicyclic conformationally constrained Smac mimetics with XIAP BIR3, a crystal structure of compound **18** in a complex with XIAP BIR3 was determined (Figure 2C).[32] This crystal structure showed that compound **18** closely mimics the Smac AVPI peptide for binding to XIAP BIR3 in both hydrogen bonding and hydrophobic interactions. Furthermore, the predicted binding model by computational modeling for compound **18** in complex with XIAP BIR3 is in excellent agreement with that revealed in the crystal structure.[32]

Zobel and colleagues from Genentech reported the design of a set of conformationally constrained Smac mimetics.[33] The most potent compound (**21**) binds to XIAP BIR3 with a K_i value

14 (K_i = 61 nM) **15** (K_i = 14.4 μM)

17 (SM-122) (K_i = 26 nM) **18** (SM-130) (K_i = 67 nM)

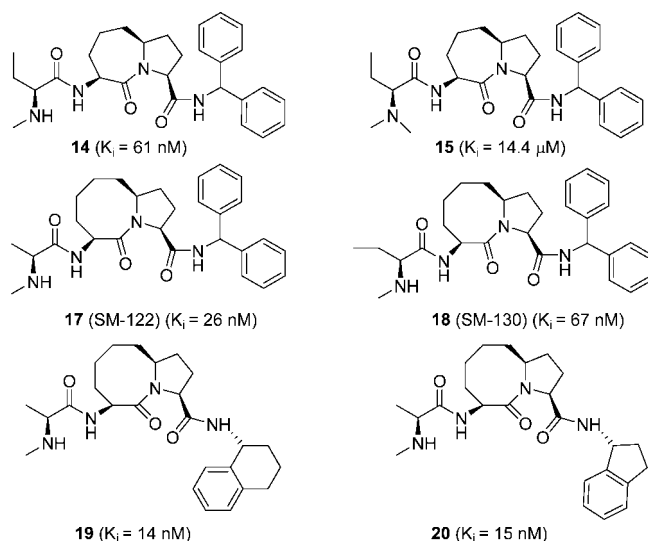**19** (K_i = 14 nM) **20** (K_i = 15 nM)

FIGURE 6. Representative potent, cell-permeable, nonpeptidic Smac mimetics and control compounds reported from the Wang laboratory in the University of Michigan and their collaborators.
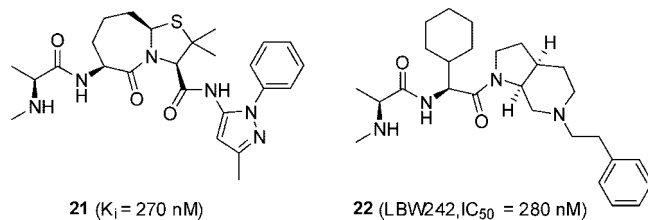

21 (K_i = 270 nM) **22** (LBW242, IC$_{50}$ = 280 nM)

FIGURE 7. Conformationally constrained Smac mimetics from Genentech and Novartis.

of 270 nM (Figure 7). Scientists from Novartis reported the design of LBW242 (compound **22**, Figure 7). Compound **22** was designed by cyclization of the third and fourth residues in the AVPI peptide and binds to XIAP BIR3 with an IC$_{50}$ value of 280 nM.[34,35]

Design of Bivalent Smac Mimetics

It has been demonstrated that the natural Smac protein forms a dimer (Figure 2A)[16,17] and binds to XIAP protein constructs containing BIR2 and BIR3 domain with a much higher affinity than the Smac AVPI peptide. Indeed, functional studies have shown that Smac protein is a much more efficient and potent antagonist than the AVPI peptide against XIAP protein containing the BIR2 and BIR3 domains in relieving the inhibition by XIAP of the activity of caspase-9 and caspase-3 and -7.[19,20] The Smac AVPI binding motif binds to both BIR2 and BIR3 domains, although with a stronger affinity to BIR3.[18] Thus small molecules designed to have two "AVPI" binding motifs may mimic the mode of action of Smac protein to target XIAP and be capable of achieving very high binding affinities to XIAP by concurrently targeting both the BIR2 and BIR3 domains in the protein.
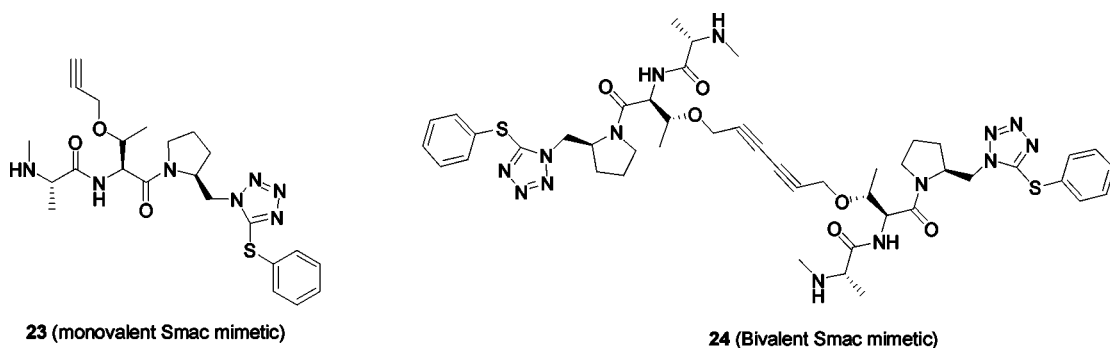
FIGURE 8. First bivalent small-molecule Smac mimetic from the laboratories of Wang and Harran at the University of Texas Southwestern Medical Center.
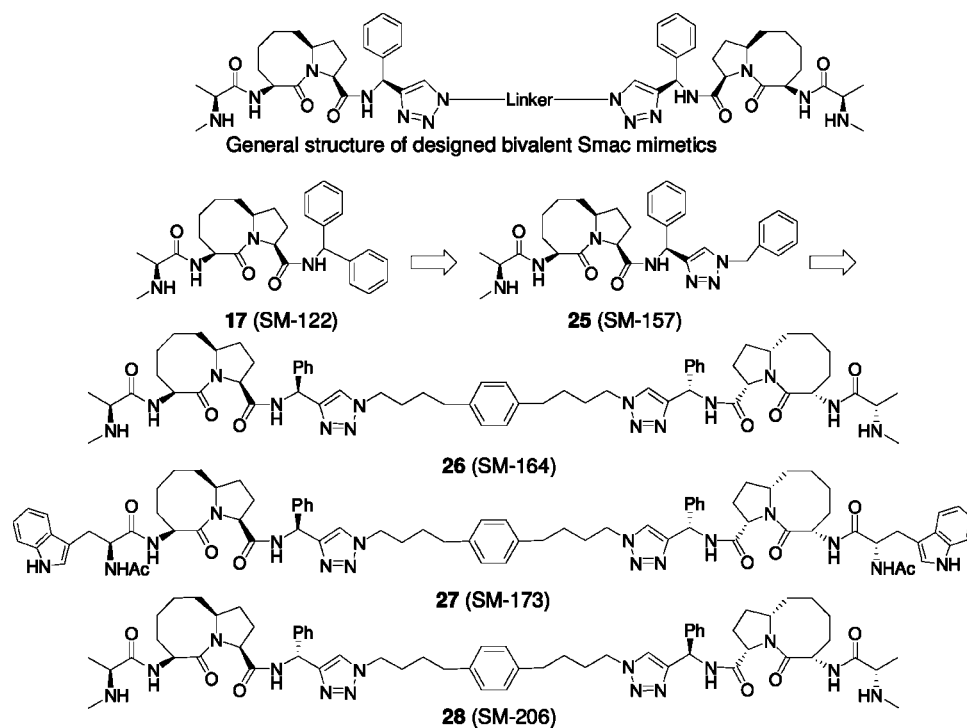

FIGURE 9. Bivalent small-molecule Smac mimetics from the Wang laboratory at the University of Michigan.

The Wang and Harran laboratories from the University of Texas Southwestern Medical Center reported the discovery of such a bivalent small molecule Smac mimetic in 2004.[36] In biochemical binding assays, this bivalent Smac mimetic **24** binds to recombinant XIAP BIR3 protein with an affinity comparable to that of its monovalent counterpart, compound **23**, and the AVPF peptide (Figure 8). However, when the XIAP full-length protein was used, it was estimated that compound **24** may have an affinity higher than Smac protein, whose estimated K_d value is 0.3 nM. It also relieves the caspase-3 inhibition by XIAP with a potency similar to that of Smac protein but much higher than that of the corresponding monovalent Smac mimetics.[36] Recently, Nikolovska-Coleska et al., using a newly established fluorescence-polarization based assay,

determined that compound **24** binds to XIAP containing both the BIR2 and BIR3 domains with a K_i value <0.7 nM.[38]

Sun et al. recently reported the structure-based design of nonpeptidic, bivalent Smac mimetics based upon conformationally constrained monovalent Smac mimetics, and they characterized in detail the interaction of both monovalent and bivalent Smac mimetics with different XIAP protein constructs (Figure 9).[37]

Although compound **17** (SM-122) was initially designed to target the XIAP BIR3 domain, modeling predicted that it may also bind to XIAP BIR2. Using the surface plasmon resonance method and a biotinylated analogue of SM-122, it was determined that SM-122 indeed binds to XIAP BIR2 with an IC_{50} value of 5 μM. For the design of bivalent Smac mimetics, it

was critical to identify sites appropriate for tethering. Computational modeling indicated that the pro-*R* phenyl ring in SM-122 inserts into a hydrophobic pocket in both BIR2 and BIR3 domains, while the pro-*S* phenyl ring is exposed to solvent and has no similar interaction with the protein. This phenyl ring is therefore a suitable site for tethering two molecules of SM-122 to one another. Modeling further suggested that the pro-*S* phenyl ring may be replaced with a [1,2,3]-triazole structure without detrimental effect. Tethering two monovalent Smac mimetic molecules together can be easily accomplished with "click chemistry". Modeling analysis showed that the linker region between BIR2 and BIR3 domains of XIAP should be quite flexible, suggesting that a bivalent Smac mimetic with a relatively short linker between the two monovalent Smac mimetics could concurrently interact with both BIR domains. Based upon these considerations, a bivalent Smac mimetic **26** (SM-164) was designed and synthesized. To test whether the stereospecificity of the two triazole rings is important for binding, a stereoisomer **28** (SM-206), differing from SM-164 only in the configuration of the two chiral centers where the triazole rings are connected, was designed and synthesized. To test the specificity of SM-164, compound **27** (SM-173) was designed as an inactive control. In compound **27**, the methyl group in each monovalent binding unit was replaced by a methyl-1*H*-indole to disrupt the hydrophobic interactions at this site, and the methylamino group was replaced by an acetamido group to block hydrogen bond formation.

In order to determine the binding affinities of these designed bivalent Smac mimetics accurately, a fluorescence-polarization based assay using the XIAP protein containing both BIR2 and BIR3 domains and a fluorescently tagged bivalent Smac-based peptide tracer was developed. Compounds **17**, **26**, and **28** and the AVPI peptide were determined to bind to XIAP containing both BIR2 and BIR3 domains with IC_{50} values of 438, 1.39, 71.5, and 10 396 nM, respectively, while the designed inactive control **27** shows no appreciable binding at 100 μM. Hence, the bivalent Smac mimetic **26** is 271 times more potent than the monovalent compound **17** and >7000 times more potent than the Smac AVPI peptide. The stereoisomer **28** is 51-times less potent than compound **26**, confirming the importance of the stereospecificity. The binding data show that the bivalent Smac mimetic **26** has an extremely high binding affinity for XIAP BIR2−BIR3 protein and is much more potent than monovalent **17** and the natural Smac AVPI peptide.

Compounds **17** and **26** were evaluated for their ability to antagonize XIAP in cell-free functional assays. In these assays,

XIAP containing BIR2−BIR3 domains and the linker preceding BIR2 (residues 120−356) dose-dependently inhibits the activity of caspase-9 and caspase-3/-7 and achieves complete inhibition at 50 nM. Both compounds **17** and **26** antagonize XIAP in a dose-dependent manner and are capable of restoring the activity of caspase-9, as well as that of caspase-3 and caspase-7. Consistent with their binding affinities to XIAP, compound **26** is 100 times more potent than **17**. At a concentration equimolar to that of XIAP, compound **26** completely overcomes the inhibition of XIAP and fully restores the activity of caspase-9 and -3/-7, indicating its extremely high potency as an XIAP antagonist. In comparison, the Smac AVPI peptide at a concentration of 100 μM (2000 times the concentration of XIAP protein) is needed to completely restore the activity of caspase-9 and caspase-3/-7. The inactive control **27** has a minimal effect at 100 μM. These functional data showed that while both compounds **17** and **26** function as antagonists of XIAP, the bivalent Smac mimetic **26** is 100- and 2000-times more potent than the corresponding monovalent Smac mimetic **17** and the Smac AVPI peptide, respectively, consistent with their binding affinities with XIAP.

The mode of binding of these monovalent and bivalent Smac mimetics to XIAP containing either BIR3-only or BIR2−BIR3 domains were further investigated by analytical gel filtration using wild-type and mutated XIAP proteins and heteronuclear single-quantum correlation (HSQC) NMR spectroscopy. The analytical gel filtration and NMR data provide clear evidence that in the presence of XIAP BIR3-only protein, one bivalent Smac mimetic **26** molecule interacts with two BIR3-only molecules, causing dimerization. However, in the presence of XIAP protein containing both BIR2 and BIR3 domains, one bivalent Smac mimetic **26** molecule interacts concurrently with both BIR2 and BIR3 domains in XIAP.

Jiang and colleagues reported the synthesis and evaluation of compound **29**, a bivalent Smac peptidic ligand (Figure 10).[46] Using this compound as a tool, they demonstrated that binding of compound **29** with the BIR2 domain of XIAP effectively antagonizes inhibition of caspase-3 by XIAP. They showed further that binding of Smac protein with the BIR3 domain anchors the subsequent binding of Smac with the BIR2 domain, which in turn attenuates the caspase-3 inhibitory function of XIAP, suggesting that both Smac protein and bivalent Smac mimetics may bind to XIAP containing BIR2 and BIR3 domains in a sequential and cooperative manner.

Compound **30** was designed and employed as a cell-permeable, bivalent Smac mimetic by Varfolomeev and colleagues from Genentech to investigate the mechanism of action of Smac mimetics in induction of apoptosis.[42] Com-
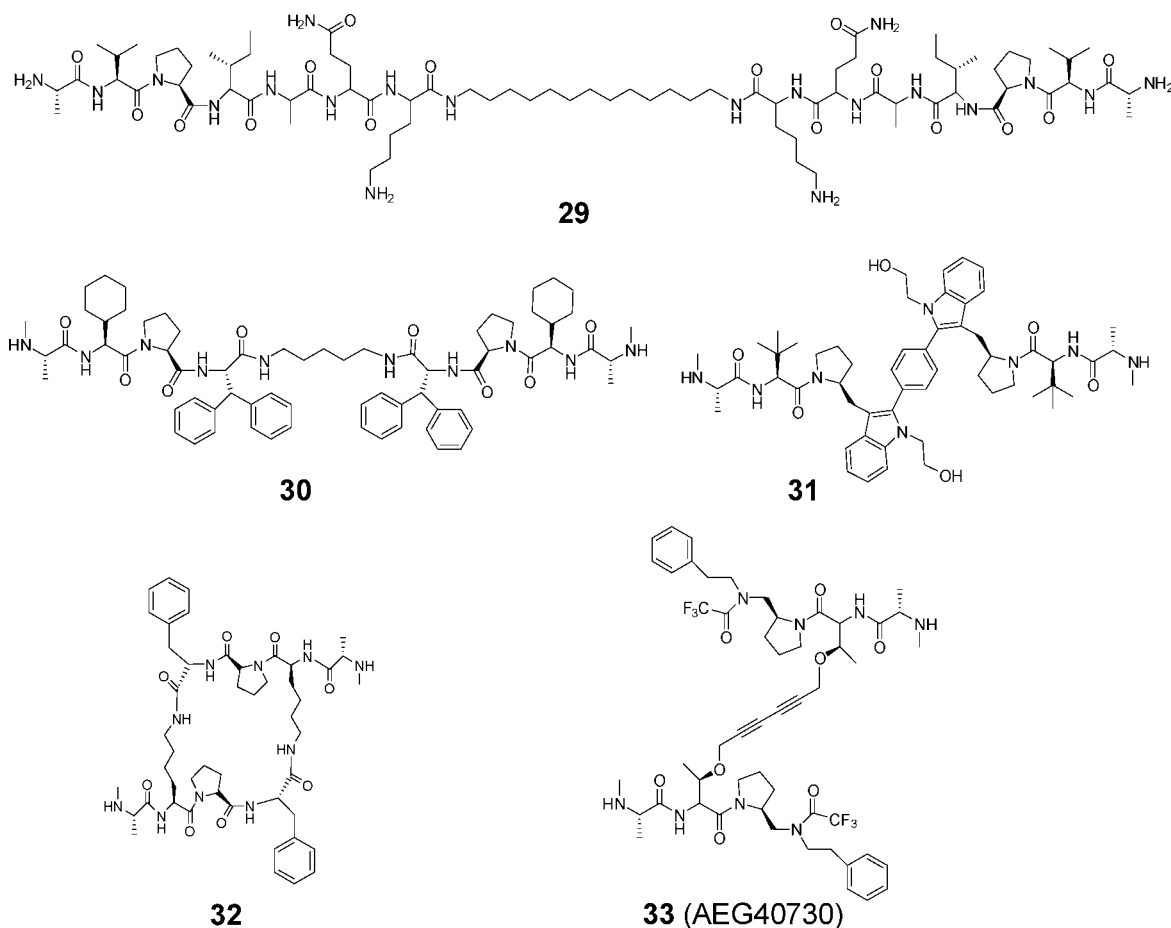
FIGURE 10. Other recently published bivalent Smac peptididomimetics.

pound **30** was determined to bind to XIAP containing both BIR2 and BIR3 domains with a K_i value of 1.30 nM.

Compound **31** is a potent and cell-permeable bivalent Smac mimetic, developed by TetraLogic Pharmaceuticals.[41] Although it was indicated to bind to XIAP with a picomolar affinity, no quantitative value was provided.[41]

Nikolovska-Coleska and her colleagues reported the design, synthesis, and evaluation of compound **32** as a cyclic, bivalent Smac mimetic.[47] They showed that compound **32** binds to XIAP containing both BIR2 and BIR3 domains with a biphasic dose−response curve, revealing two binding sites with IC_{50} values of 0.5 and 406 nM, respectively. Compound **32** binds to XIAPs containing the BIR3-only and BIR2-only domain with K_i values of 4 nM and 4.4 μM, respectively. Gel filtration experiments using wild-type and mutated XIAPs showed that **32** forms a 1:2 stoichiometric complex with XIAP containing the BIR3-only domain. However, it forms a 1:1 stoichiometric complex with XIAP containing both BIR2 and BIR3 domains, and both BIR domains are involved in the binding. Compound **32** efficiently antagonizes inhibition of XIAP in a cell-free functional assay and is >200 times more potent than

its corresponding monovalent Smac mimetic. Determination of the crystal structure of **32** in complex with the XIAP BIR3 domain confirms that it induces homodimerization of the XIAP BIR3 domain and provides a structural basis for the cooperative binding of one molecule of compound **32** to two XIAP BIR3 molecules. On the basis of this crystal structure, a binding model of XIAP containing both BIR2 and BIR3 domains and **32** was constructed, which suggested that the binding of compound **32** to XIAP blocks the binding of XIAP to caspase-3/-7.

Another recently reported cell-permeable bivalent Smac mimetic is compound **33**, designed and developed by scientists from Aegera Therapeutics in Montreal.[44] Compound **33** binds to XIAP BIR3 protein with an IC_{50} value of 100 nM.[44]

Smac Mimetics Bind Not Only to XIAP but Also to cIAP-1/-2 and ML-IAP

The design of Smac mimetics was primarily based upon the interaction between Smac and XIAP. However, Smac also binds to cIAP-1/-2 and ML-IAP. Thus, it is not surprising that

small-molecule Smac mimetics also target other IAP proteins, in addition to XIAP.

For monovalent Smac mimetics, compound **21** was shown to bind to cIAP-1, cIAP-2 and ML-IAP with K_i values of 50, 130 and 50 nM, respectively.[33] Compound **17** binds to cIAP-1 and cIAP-2 proteins with very high affinities, having K_i values of 2.7 and 1.9 nM, respectively.[45]

For bivalent Smac mimetics, compound **26** was determined to bind to cIAP-1 protein containing BIR2 and BIR3 domains with a K_i value of 0.3 nM and to cIAP-2 BIR3 protein with a K_i value of 1.1 nM.[45] Compound **30** was shown to bind to cIAP-1 containing both BIR2 and BIR3 domains with a K_d value of 0.46 nM.[42] It was also indicated that compound **31** binds to cIAP-1 with a picomolar affinity, but the precise value was not reported.[41] Compound **33** binds to cIAP-1 and cIAP-2 BIR3 proteins with IC_{50} values of 17 and 34 nM, respectively.[44] Although the precise binding affinities of the bivalent Smac mimetic **24** to cIAP-1/-2 were not reported, it was shown that its biotinylated analogue can pull down endogenous cIAP-1 and cIAP-2 in cell lysates, in addition to XIAP.[36]

Taken together, these biochemical data clearly show that both monovalent and bivalent Smac mimetics bind with high affinities to not only XIAP but also other IAP proteins, including cIAP-1 and cIAP-2 proteins.

Smac Mimetics as New Anticancer Agents

A number of studies have demonstrated that Smac-based cell-permeable peptides are effective in sensitizing cancer cells to a variety of anticancer agents.[25–27] Subsequent studies showed that both monovalent and bivalent Smac mimetics as single agents can inhibits cell growth and induce apoptosis in cancer cells.[28,31–37]

Oost and colleagues showed that peptide-mimetic **3** inhibits cell growth in seven different cell lines with diverse tumor types with IC_{50} values ranging from 7 nM to 2 μM.[28] These include breast cancer cell lines BT-549 and MDA-MB-231, leukemia cell line HL-60, melanoma cell line SK-MEL-5, renal cancer cell line RXF-393, ovarian cancer cell line SK-OV-3, and non-small-cell lung cancer cell lines NCI-H23 and NCI-H522. Zobel and colleagues showed that compound **21** inhibited cell growth with IC_{50} values of 100 nM and 2 μM in the MDA-MB-231 breast cancer cell line and A-2058 melanoma cell line, respectively. These Smac mimetics were shown to also effectively induce cell death and apoptosis in cancer cells in a caspase-dependent manner.

The bivalent Smac mimetic **24** was initially shown to potentiate the activity of TRAIL and tumor necrosis factor α (TNFα) but had no activity as a single agent in the T98G

glioma cell line.[36] In a subsequent study, it was demonstrated to be effective in cell growth inhibition in ~25% of human non-small carcinoma cell lung cancer cell lines and achieve IC_{50} values in the nanomolar range in 14% of cancer cell lines.[40] Compound **24** also induces robust cell death at 100 nM in these sensitive cell lines. Furthermore, it effectively inhibits tumor growth in the HCC461 xenografts in mice and causes tumor regression in 40% of treated animals.[40]

Both conformationally constrained monovalent and bivalent Smac mimetics reported by the Wang laboratory at the University of Michigan and their collaborators were shown to be effective in inhibition of cell growth and induction of apoptosis in cancer cell lines.[31,32,37] For example, monovalent **17** (SM-122) potently inhibits cell growth and induce apoptosis in a number of cancer cell lines, including the MDA-MB-231 breast cancer cell line, SK-OV-3 ovarian cancer cell line and HL-60 leukemia cells. Compound **26** (bivalent SM-164) achieves IC_{50} values of 1 nM or less in inhibition of cell growth in the MDA-MB-231, SK-OV-3 and HL-60 cell lines and also effectively induces apoptosis in these cancer cell lines at concentrations as low as 1 nM.[37,45] SM-164 is capable of inducing tumor regression in the MDA-MB-231 xenografts in mice and shows no or little toxicity to animals at effective dose schedules.[45]

These *in vitro* and *in vivo* data from a number of laboratories using different Smac mimetics have provided strong evidence that Smac mimetics may have a great therapeutic potential for the treatment of human cancer not only in combination with other therapeutic agents but also as single agents.

Potential Advantages and Disadvantages of Monovalent and Bivalent Smac Mimetics as New Anticancer Agents

As potential drug candidates, there are advantages and disadvantages associated with monovalent and bivalent Smac mimetics. Monovalent Smac mimetics are less potent than their corresponding bivalent Smac mimetics.[36,37] However, monovalent Smac mimetics, with a molecular weight of ~500, possess many desirable pharmacological properties as potential drug candidates. Properly designed monovalent Smac mimetics can achieve good oral bioavailability. Many molecularly targeted small-molecule anticancer drugs developed in the last decade are given repeatedly in the clinic for a prolonged period of time, for example daily for 3–4 weeks. An orally bioavailable Smac mimetic will provide an important advantage to its clinical development. Bivalent Smac mimetics have been shown to be 100–1000 times more potent

than their monovalent counterparts[37] and thus could be potentially far more efficacious. However, since bivalent Smac mimetics have a molecular weight exceeding 1,000, such compounds may be expected to have very low oral bioavailability and will have to be administered by other routes of administration, such as intravenous dosing, a potential disadvantage if the drug must be given to patients frequently.

Mechanism of Action of Smac Mimetics in Apoptosis Induction

The availability of potent, specific, and cell-permeable Smac mimetics has provided powerful biological tools with which to gain important insights into apoptosis regulation by IAP proteins.

Using cell-permeable Smac-based peptides, it was shown that such compounds can enhance the activity of chemotherapeutic agents and TRAIL.[25–27] Such data were expected because XIAP is a potent inhibitor of apoptosis and Smac-based compounds should antagonize XIAP, thus enhancing the ability of chemotherapeutic agents and TRAIL to induce apoptosis in tumor cells. However, using a set of very potent Smac peptidomimetics, Oost and colleagues showed that such compounds can effectively inhibit cell growth and induce apoptosis as single agents in a panel of cancer cell lines.[28] The single agent activity in cancer cells has been also observed subsequently for other Smac mimetics with different chemotypes, for both monovalent and bivalent Smac mimetics.[31,32] However, for a number of years, it was unclear how Smac mimetics alone can induce apoptosis in certain tumor cell lines without the need of an external apoptosis stimulus. This mystery has now been solved by several elegant and independent studies.[40–44]

As indicated above, Smac mimetics bind not only to XIAP but also to cIAP-1 and cIAP-2, proteins with very high affinities in biochemical assays. Several recent studies have clearly demonstrated that both monovalent and bivalent Smac mimetics cause rapid degradation of cIAP-1/-2 proteins.[40–44] Binding of Smac mimetics to cIAP-1/-2 induces autoubiquination of cIAP-1/-2 proteins, followed by protein degradation in a proteasomal-dependent manner.[40–44] In sensitive tumor lines, degradation of cIAP-1/-2 by Smac mimetics induces neuronal factor κB (NF-κB)-stimulated production of TNFα. TNFα promotes formation of an receptor-interacting serine-threonine kinase 1 (RIPK1)-dependent caspase-8-activating complex upon removal of cIAP-1/-2, leading to activation of caspase-8 and -3/-7, and ultimately apoptosis.[40–44] These studies established that induction of cIAP-1/-2 degradation is a key early event in apoptosis induction by Smac mimetics, and cIAP-1 and cIAP-2 are critical cellular targets for Smac mimetics. Furthermore, it was shown that while caspase-3 plays a critical role in apoptosis induction by Smac mimetics in sensitive cancer cell lines, caspase-9 appears to play a modest role.[40]

Interestingly, although Smac mimetics were designed based upon the interaction of XIAP and Smac, the role of XIAP in apoptosis induction by Smac mimetics was not well defined in these studies.[40–44] To address this question, Lu and colleagues have investigated the role of XIAP and cIAP-1/-2 in apoptosis induction by monovalent mimetic SM-122 (compound **17**) and bivalent mimetic SM-164 (compound **26**). They showed that removal of cIAP-1/-2 by Smac mimetics or small-interfering RNA is not sufficient for robust TNFα-dependent apoptosis induction and that XIAP plays a critical role in inhibiting apoptosis induction. Although SM-164 is slightly more effective than SM-122 in induction of cIAP-1/-2 degradation, SM-164 is 1000 times more potent than SM-122 as an inducer of apoptosis in tumor cells, an observation that can be attributed to its much higher potency in binding to and antagonizing XIAP. SM-164 induces rapid cIAP-1 degradation and strong apoptosis in the MDA-MB-231 xenograft tumor tissues and achieves tumor regression but has no toxicity in normal mouse tissues. These data provide strong evidence that Smac mimetics induce apoptosis in tumor cells by concurrently targeting cIAP-1/-2 and XIAP, suggesting that XIAP and cIAP-1/-2 are important cellular targets for Smac mimetics.

Summary

Since the discovery in 2000 of the Smac protein, there has been an enormous interest in academic laboratories and pharmaceutical companies in the design of small-molecule Smac mimetics. This task is particularly challenging because it involves targeting protein–protein interactions. Nevertheless, intense research has now generated potent, specific, cell-permeable small-molecule peptidomimetics and nonpeptidic mimetics. A structure-based strategy has been employed to develop monovalent Smac mimetics designed to mimic the Smac AVPI binding motif and so target the XIAP BIR3 domain. Such compounds were found to achieve not only high affinities to XIAP BIR3 but also high affinities to cIAP-1, cIAP-2 and ML-IAP proteins. Compounds that contain two "AVPI" binding motifs have also been designed and evaluated. It was found that such bivalent Smac mimetics bind to XIAP protein containing both BIR2 and BIR3 domains with an extremely high affinity, exceeding that of Smac protein. It has been clearly

shown that bivalent Smac mimetics achieve such high affinities by concurrently targeting both the BIR2 and BIR3 domains in XIAP.

IAP proteins potently suppress apoptosis, and Smac protein promotes apoptosis by antagonizing IAP proteins. Hence, it was originally thought that while Smac-based compounds could effectively sensitize cancer cells to other therapeutic agents in apoptosis induction by targeting IAP proteins, they may have limited activity as single agents. Subsequent studies from a number of laboratories have shown that both monovalent and bivalent Smac mimetics are capable as single agents of inducing apoptosis in some but not all human cancer cell lines. It was further shown that bivalent Smac mimetics can induce robust apoptosis in cancer cells at concentrations as low as 1 nM and are 100−1000 times more potent than the corresponding monovalent Smac mimetics. Several recent independent studies have shown that Smac mimetics induce apoptosis in tumor cells by targeting cIAP-1/-2, causing rapid degradation of these proteins. Degradation of cIAP-1/-2 activates NF-κB, in turn, induces the production of TNFα. TNFα promotes formation of an RIPK1-dependent caspase-8-activating complex, leading to activation of caspase-8 and -3/-7, and ultimately to apoptosis. A major surprise from these recent mechanistic studies is that while caspase-3 was confirmed to play a major role in apoptosis induction by Smac mimetics, caspase-9 appears to play only a modest role. A recent study further shows that Smac mimetics induce apoptosis in tumor cells by targeting not only cIAP-1/2 but also XIAP.[45] These important insights into the regulation of apoptosis by IAP proteins were achieved, to a large extent, because of the availability of potent, cell-permeable, small-molecule Smac mimetics, highlighting the important interface between chemistry and biology to advance our understanding on the regulation of apoptosis.

To date, at least one Smac mimetic has been advanced into clinical development, although the chemical structure of this compound has not been disclosed. Several other Smac mimetics are in an advanced preclinical development stage and are expected to enter human clinical testing for the treatment of cancer.

BIOGRAPHICAL INFORMATION

Haiying Sun received his B.S. in Chemistry and M.S. in Organic Chemistry from Lanzhou University in 1991 and 1997, respectively. He gained his Ph.D. in Organic Chemistry from Shanghai Institute of Organic Chemistry in 2000 with Professor Dawei Ma. Dr. Sun did his first postdoctoral training with Professor Alan P. Kozikowski at Georgetown University from 2000-2002. He joined Dr. Shaomeng Wang's group at the University of Michigan in 2002 and did his second postdoctoral training from 2002-2003. He was a Research Investigator between 2003 and 2008 and is currently a Research Assistant Professor in the Department of Internal Medicine at the University of Michigan. His research interest is to design and synthesize small molecular modulators of proteins related to crucial physiological processes with the purpose to develop new therapies for the treatment cancer and other human diseases.

Zaneta Nikolovska-Coleska received her B.S. in Pharmacy from University Ss. Cyril and Methodius, Skopje, Republic of Macedonia, in 1987 and her Ph.D. in Pharmaceutical Chemistry from the same University in 1999 with Professor Kiril Dorevski and Professor Tomaz Solmajer from the National Institute of Chemistry, Slovenia. Dr. Nikolovska-Coleska did her postdoctoral training in drug discovery at the University of Michigan between 2001 and 2004 with Professor Shaomeng Wang. Dr. Nikolovska-Coleska was a Research Investigator and a Research Assistant Professor in Dr. Wang's laboratory in the Department of Internal Medicine, University of Michigan, from 2004-2008. Currently Dr. Nikolovska-Coleska is starting her new position as a tenured Assistant Professor in the Department of Pathology, University of Michigan. Dr. Nikolovska-Coleska is an author of 44 peer-reviewed publications and an inventor on nine patents.

Chao-Yie Yang worked with Professor Stephen J. Klippenstein on variational transition state theory at Case Western Reserve University and received his doctoral degree in 1996. After his postdoctoral works with Professor Stephen K. Gray at National Argonne Laboratory and Professor Peter J. Rossky at University of Texas at Austin, he moved to the University of Michigan in 2001 to work with Professor Shaomeng Wang and focused on the research of structural biology and drug design. He is currently a Research Investigator at the University of Michigan.

Jianfeng Lu received his Ph.D. in Immunotherapy for Liver Cancer from Peking University in 1998. Dr. Lu has been a Clinical Professor at Zhengzhou University in China since 2003. Dr. Lu is a visiting scholar, and is in postdoctoral training with Dr. Shaomeng Wang in the Comprehensive Cancer Center at the University Michigan, Ann Arbor, MI, since 2004. Dr. Lu is an author of 40 publications, three book chapters and an inventor on two issued patents and patent applications. Dr. Lu has trained five graduate students.

Su Qiu, M.D. Dr. Qiu received her B.S. at Hunan Xiangya University, in P. R. of China in 1983 and her M.D. at the University of Berne, Switzerland in 1996. Dr. Qiu did her post doctoral training at Zurich University, Switzerland in 1996-1997. Dr. Qiu was

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Dongguang Qin received his B.S. in Chemistry from Fudan University in 1998 and his Ph.D. in Organic Chemistry from Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences in 1998 with Professor Zhu-Jun Yao. Dr. Qin did his postdoctoral research focused on the design, synthesis and development of new anticancer drugs at the University of Michigan with Professor Shaomeng Wang between 2004 and 2007. Dr. Qin was the Principle Investigator at Chengdu Di'ao Pharmaceutical Group between 2007 and 2008. Dr. Qin is currently the executive vice president and co-founder of Helix&Bond Pharmaceutical Inc., Shanghai, China.

Qian Cai received his B.S. in Chemistry from Nankai University in 2001 and his Ph.D. in Organic Chemistry from Shanghai Institute of Organic Chemistry in 2006 with Professor Dawei Ma. Dr. Cai joined Professor Shaomeng Wang's group for his postdoctoral training in drug design at the University of Michigan, Ann Arbor, MI, where he has been since 2006. Dr Cai's interests are development of new synthetic methods and their application in target synthesis for drug discovery.

Shaomeng Wang received his B.S. in Chemistry from Peking University in 1986 and his Ph.D. in Chemistry from Case Western Reserve University in 1992 with Professor Gilles Klopman. Dr. Wang did his postdoctoral training in drug design at the National Cancer Institute, NIH, between 1992-1996 with Dr. Bill Milne. Dr. Wang was Assistant Professor at Georgetown University from 1996-2000 and Associate Professor from 2000-2001. Dr. Wang was a tenured Associate Professor between 2001-2006 at the University of Michigan and is currently Professor of Medicine, Pharmacology and Medicinal Chemistry. Dr. Wang serves as the Co-Director of the Molecular Therapeutics Program at the University of Michigan Comprehensive Cancer Center and holds the Warner-Lambert/Parke-Davis Professorship in Medicine at the University of Michigan Medical School. Dr. Wang is an author of 170 peer-reviewed publications and an inventor on more than 25 issued patents and patent applications. Dr. Wang has trained more than 25 graduate students and postdoctoral fellows. Dr. Wang is a co-founder of Ascenta Therapeutics, which focuses on development of apoptosis-based therapies for cancer treatment.

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